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Dissociation of the Anti-Hapten and Anti-Carrier Responses of Mice Injected with Dinitrophenylated Lipopolysaccharide

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The quantitative and qualitative nature of the antibody responses of euthymic (normal, RML) and athymic (nude) mice injected with dinitrophenylated (DNP) lipopolysaccharide (LPS) was evaluated. Antibody responses to both the haptenic (DNP) and carrier (LPS) determinants were measured. On a quantitative basis, RML and nude mice stimulated with DNP-LPS produced only primary anti-DNP responses, whereas both primary and secondary anti-LPS responses were elicited by this conjugate. The failure of DNP-LPS to trigger secondary anti-DNP responses was not dependent on the amount of DNP-LPS given in the primary or secondary doses and could not be overcome by repeated injections of DNP-LPS. Also, the anti-DNP responses of RML mice injected repeatedly with DNP-LPS were restricted to immunoglobulin M antibodies, whereas both immunoglobulin M and G anti-LPS responses were elicited. Nude mice also produced immunoglobulin G antibodies to the LPS determinants. These data showed a dissociation of the anti-hapten and anti-carrier antibody responses and suggested that different immunological signals were functioning in the respective anti-DNP and anti-LPS responses.

Historically, several different polymeric antigens, such as lipopolysaccharide (LPS) (1), polyvinylpyrrolidone (1), type III pneumococcal polysaccharide (19), levan (20), and polymerized flagellin (10) were shown to stimulate antibody production by B cells without help by T cells. These T cell-independent antigens were instrumental in defining some of the requirements for activation of B cells, in terms of both nonspecific mitogenic activation and elicitation of specific antibody responses. For example, native and modified LPS and native protoplasmic polysaccharide were used to show a functional separation of immunological signals required to elicit primary and to trigger secondary anti-LPS responses (27, 31, 32). Furthermore, others have shown that responses to haptens coupled to different T cell-independent carriers were T cell independent (6-9, 11, 17, 28). With the exception of dinitrophenyl (DNP)-Ficoll (21), responses elicited by these conjugates were restricted to primary immunoglobulin M (IgM) responses; even repeated injections of the homologous conjugate would not elicit a true secondary anti-hapten response (7, 11). In the studies cited above the anti-hapten responses to conjugates with T cell-independent carriers were well characterized; however, antibody responses to antigenic determinants of the T cell-independent carrier molecules were not measured.

The present paper describes the antibody responses of euthymic (normal) and athymic (nude) mice injected with the DNP hapten conjugated to a T cell-independent carrier, bacterial LPS. Measurement of the antibody responses to both the haptenic and carrier determinants revealed important quantitative and qualitative differences. DNP-LPS elicited only primary IgM responses to the DNP determinant and both IgM and IgG responses to the carrier determinants. The marked discrepancies in the responses to the hapten and carrier determinants suggested that different immunological signals were functional in the respective anti-DNP and anti-LPS responses.

MATERIALS AND METHODS

Mice. Outbred mice of both sexes were obtained from the Rocky Mountain Laboratory (RML), Hamilton, Mont. These are from the N.NIH(s) colony that has been described elsewhere (24). Congenitally athymic nude mice were bred in our laboratory and were the offspring of heterozygous animals obtained by crossing nude males with RML females. To improve their general health, nude mice received water containing oxytetracycline (Pharmaceutical Co., Krakov, Poland) and metronidazole (Flagyl, Searle and Co., Columbus, Ohio). Postmortem examinations confirmed that the nude mice used in the experiments were athymic.

Antigens. Whole cells of *Escherichia coli* O113 were grown as described previously (13). The bacteria were washed three times in phosphate-buffered saline

(PBS; 0.15 M NaCl, 0.0033 M PO_4, pH 7.2), suspended in PBS, and killed by placing the suspension in a boiling water bath for 1 h. Merthiolate (powder no. 20; Eli Lilly & Co., Indianapolis, Ind.) was added to the killed suspension to a final concentration of 0.01% and, after a sample was removed for determination of dry weight, the suspension was stored at 4°C. This vaccine was the LPS used for immunizing mice.

The bacterial cells were dinitrophenylated by modification of a procedure described by Gander and Rudbach (14). Approximately 100 mg of heat-killed bacteria was suspended in 5.0 ml of 0.21% $NaHCO_3$. Next, 0.5 ml of 1-fluoro-2,4-dinitrobenzene (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added, and the mixture was stirred slowly in the dark for 1 h at room temperature (20 to 22°C). The cells were allowed to stand overnight at 4°C in the dark, collected by centrifugation, and washed with PBS until the supernatant fluid was colorless. These derivatized cells of *E. coli*, designated DNP-LPS, were suspended in PBS containing 0.01% Merthiolate, quantified, and stored at 4°C.

Dinitrofluorobenzene reacts readily with most free amino groups, and it was assumed that ethanolamine residues in LPS of the outer membrane were the sites for attachment of the dinitrophenyl groups. In fact, other experiments with haptens conjugated directly to extracted LPS gave results which were similar to those presented for DNP conjugated to the bacterial cells (11; Von Eschen, unpublished observations). Therefore, the immunological responses presented in this paper truly represent those of DNP-LPS.

Immunoassays. Standard passive hemagglutination procedures, employing sheep erythrocytes coated with either LPS (26) or DNP (30) as indicator cells, were used to determine the titers of humoral antibodies specific for the LPS or DNP determinant. Antibody titers are expressed as values of x derived from the equation $x = \log_2 (HD/2)$, where HD is the reciprocal of the highest dilution of serum which produced hemagglutination of sensitized sheep erythrocytes (18). Thus, the titer is the tube number of the endpoint when the first tube contains a 1/4 dilution of antiserum. To facilitate calculations, sera which gave no hemagglutination at the lowest dilution tested were arbitrarily assigned a titer of 0, i.e., a dilution of 1/2.

In some cases a higher initial dilution of antiserum was required for immunoglobulin classification experiments (see Tables 4 and 5 and Fig. 3). In these assays titers are expressed as values of x derived from the equation $x = \log_2 (HD/5)$. Thus, the titer in these experiments is the tube number of the endpoint, when the first tube contains a 1/10 dilution of antiserum. Sera which gave no hemagglutination at the lowest dilution tested in these special assays were arbitrarily assigned a titer of 0, i.e., a dilution of 1/5.

Treatment of sera with 2-ME. To destroy the hemagglutinating activity of IgM antibodies (3, 4, 12), some sera were treated with 2-mercaptoethanol (2-ME; Sigma Chemical Co., St. Louis, Mo.) as described by Rosenblatt and Johnson (25). To 0.9 ml of serum diluted 1/10 in PBS, 0.1 ml of 1.0 M 2-ME was added. After 4 h of incubation at 20 to 22°C, the serum was dialyzed for 16 to 24 h at 4°C against several changes of PBS. Control sera were treated identically, except that 0.1 ml of PBS instead of 2-ME was added to the sera. After dialysis, the sera were tested for the presence of specific antibodies by the passive hemagglutination assays described above. In some cases, iodoacetamide (Calbiochem, La Jolla, Calif.) was added to the 2-ME-treated sera to prevent spontaneous reassociation of the IgM. However, as reported previously (25), little, if any, difference was noted in the hemagglutination titers of sera treated with 2-ME whether or not iodoacetamide was added.

RESULTS

Kinetics of the appearance of antibodies in RML mice injected with DNP-LPS. To determine the time of appearance of anti-DNP and anti-LPS globulins in mice injected with DNP-LPS, groups of RML mice were injected intravenously once (day 0) or twice (days 0 and 21) with 50 μg of DNP-LPS and were bled on succeeding days. Typical primary responses, characterized by a relatively lower peak titer of antibody followed by a rapid decline in antibody, were produced to both the DNP (hapten) and the LPS (carrier) immunodeterminants after a single injection of DNP-LPS (Fig. 1). A second injection of DNP-LPS stimulated an antibody titer that was fourfold higher than that seen in the primary response to the LPS determinant. This higher peak titer and its prolonged maintenance, when compared with that resulting from a primary injection of LPS, suggested that this was, indeed, a secondary response to the LPS. In contrast, the anti-DNP response after two injections of DNP-LPS in this and subsequent experiments did not appear quantitatively or qualitatively to be a secondary response.

Failure of DNP-LPS to trigger a secondary anti-DNP response in RML mice. Data compiled from the results of several experiments (Table 1) confirmed that the anti-DNP responses of RML mice after one or two injections of 50 μg of DNP-LPS were not significantly different ($P > 0.5$ for the anti-DNP responses of group B versus group C). Analysis of group D revealed that the anti-DNP titer after two injections of DNP-LPS was not due to residual antibodies remaining after the primary injection of DNP-LPS. Therefore, mice primed with DNP-LPS and challenged 21 days later with DNP-LPS made a second primary response to the DNP determinant. On the other hand, the antibody titer to the carrier determinant (LPS) after two injections of DNP-LPS was significantly higher than the primary anti-LPS titer ($P < 0.005$ for the anti-LPS titers of group B versus group C). Finally, the titers of mice in groups E and F of Table 1 showed that the anti-DNP globulins elicited by DNP-LPS were not the result of polyclonal activation of antibody

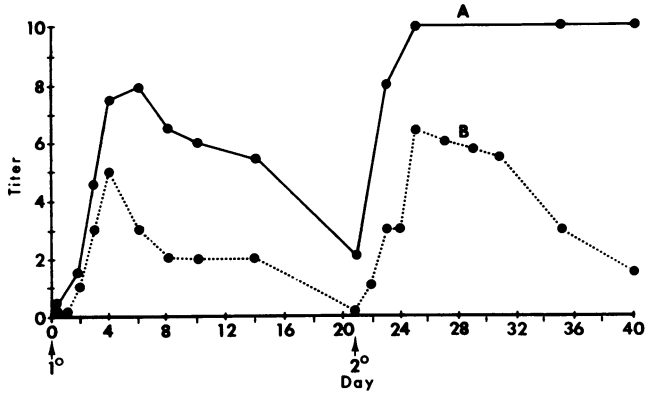

FIG. 1. *Kinetics of the anti-DNP and anti-LPS responses of RML mice injected with DNP-LPS. Each point represents the titer of sera pooled from five mice per group. A, Anti-LPS titers of mice injected intravenously with 50 μg of DNP-LPS on days 0 and 21; B, anti-DNP titers of sera from the same mice; 1°, primary injection; 2°, secondary injection.*

TABLE 1. *Antibody responses of RML mice injected with 50 μg of DNP-LPS or with 50 μg of LPS (carrier)*

Group	Injection on day:		Antibody titer[a]	
	0	21	Anti-DNP	Anti-LPS
A	None	None	0.0	0.0
B	None	DNP-LPS	4.4 (2.5–6.1)	7.2 (5.6–8.0)
C	DNP-LPS	DNP-LPS	4.3 (3.0–7.0)	10.5 (9.2–12.0)
D	DNP-LPS	None	2.0	2.0
E	None	LPS	1.0	8.0
F	LPS	LPS	1.0	11.0

[a] Antibody titer (determined on day 25) of sera pooled from five mice per group from a single experiment, except for groups B and C, in which geometric mean titers were calculated from data from separate experiments (range in parentheses).

synthesis stimulated by the LPS. Results of other experiments (data not shown) showed that the failure of DNP-LPS to trigger a secondary anti-DNP response was not due to the presence of antibodies directed against the LPS carrier and could not be accounted for by antigenic competition between the DNP and LPS determinants. In conclusion, the antibody responses to DNP-LPS were unique in that this conjugate elicited secondary responses to the carrier determinant (LPS) and, at the same time, stimulated only primary responses to the hapten (DNP).

Antibody responses of nude mice to DNP-LPS. It was noted above that DNP-LPS stimulated the production of only primary anti-DNP responses in RML mice. The antibody responses of congenitally athymic nude mice to DNP-LPS were examined to determine whether T cells were required for the immunological responses to this conjugate. The data (Table 2) showed that: (i) DNP-LPS elicited both anti-DNP and anti-LPS responses in nude mice, and (ii) nude mice injected twice with DNP-LPS made primary anti-DNP responses and secondary anti-LPS responses. Therefore, both euthymic and athymic mice gave similar responses to DNP-LPS.

Antibody responses of RML mice after repeated injections of DNP-LPS. In a final attempt to produce an anti-DNP response higher than that elicited by a primary dose of DNP-LPS, groups of RML mice were injected one, two, three, or four times, at 10-day intervals, with 50 μg of DNP-LPS; humoral antibodies against DNP and LPS were measured 4 days after the last injection of antigen. The anti-DNP titer could not be increased above that after a primary injection (Fig. 2). In these same animals, the second injection of DNP-LPS elicited a secondary response to LPS, but no further increases in titer could be produced by subsequent injections of DNP-LPS. These data corroborated the evidence which indicated that DNP-LPS could stimulate only primary antibody responses to DNP.

Effect of varying the primary and secondary doses of DNP-LPS on the antibody responses. The conclusion that DNP-LPS failed to trigger a secondary anti-DNP response was based on the results of experiments in which

TABLE 2. *Antibody responses of nude mice injected with 50 μg of DNP-LPS or with 50 μg of LPS*

Group	No. of mice	Injection on day:		Antibody titer[a]	
		0	14	Anti-DNP	Anti-LPS
A	4	None	None	0.0 (0.0)	0.0 (0.0)
B	7	None	DNP-LPS	4.1 (3.0–5.0)	7.0 (5.0–8.0)
C	4	DNP-LPS	DNP-LPS	3.5 (3.0–4.0)	10.0 (9.0–11.0)
D	3	None	LPS	0.0 (0.0)	5.0 (5.0)

[a] Geometric mean titer (determined on day 19) calculated from titers of individual mice in each group (range in parentheses).

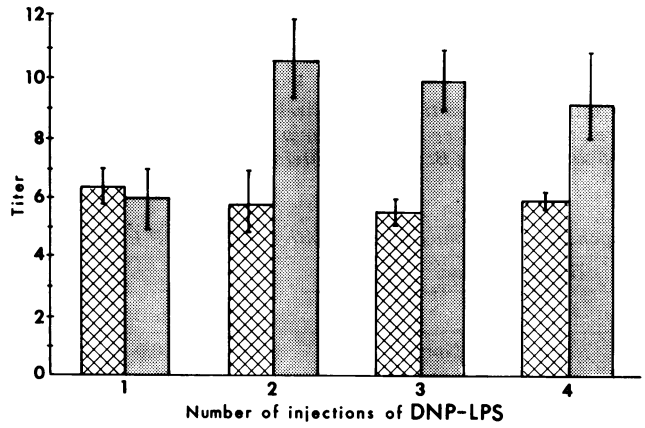

FIG. 2. *Antibody responses of RML mice to DNP (cross-hatched bars) and to LPS (spotted bars) after repeated injections of 50 μg of DNP-LPS. An interval of 10 days was used between injections. The height of the bars indicates the geometric mean peak humoral antibody titers at 4 days after the last injection of DNP-LPS (calculated from individual titers of five mice per group). The ranges of titers for each group are shown by the vertical lines.*

50 μg of DNP-LPS was used as the standard immunogenic dose. The possibility remained that different doses of DNP-LPS could elicit a secondary response. Therefore, groups of mice were injected primarily and secondarily with differing doses of DNP-LPS in a "square-titration." The anti-DNP and anti-LPS titers were determined for the sera of these mice (Table 3). In all cases, with one spurious exception, the mice did not make secondary anti-DNP responses. Therefore, the general conclusion that secondary anti-DNP responses could not be triggered by DNP-LPS appeared to be valid.

Qualitative nature of antibodies after repeated injections of DNP-LPS. Additional evidence for stimulation of only a primary response to DNP was the qualitative nature of both the anti-DNP and anti-LPS globulins produced after repeated injections of DNP-LPS. As described above (Fig. 2), repeated injections of DNP-LPS stimulated only primary anti-DNP responses. Furthermore, no 2-ME-resistant antibodies specific for DNP were present in the sera of RML mice that were injected repeatedly with DNP-LPS (Table 4). In contrast, repeated injections of DNP-LPS stimulated secondary responses as well as IgG antibodies specific for the LPS determinant.

TABLE 3. *Effect of varying the secondary dose of DNP-LPS on the antibody titers of RML primed 21 days before with different amounts of DNP-LPS*[a]

Primary dose of DNP-LPS (μg)	Anti-DNP titer with secondary dose of DNP-LPS (μg):				Anti-LPS titer with secondary dose of DNP-LPS (μg)			
	10^{-2}	10^{0}	10^{1}	10^{3}	10^{-2}	10^{0}	10^{1}	10^{3}
0	2	5	5	5	5	6	7	7
10^{-6}	2	5	4	5	6	8	7	7
10^{-2}	1	5	5	6	6	9	9	10
10^{0}	0	4	5	4	7	8	9	10
10^{1}	4	5	4	5	5	8	8	11
10^{3}	0	4	5	8	5	7	8	12

[a] Anti-DNP and anti-LPS titers on sera that were pooled from five mice per group.

Qualitative changes in antibodies to LPS not dependent on T cells. It was of interest to determine whether T cells were required for the production of IgG antibodies to the LPS determinants. Therefore, RML and athymic nude mice were injected repeatedly with 50 μg of LPS. 2-ME-resistant, hemagglutinating antibodies

TABLE 4. *Qualitative nature of the anti-DNP and anti-LPS responses of RML mice injected repeatedly with DNP-LPS*

Group	No. of injections of DNP-LPS[a]	Anti-DNP titer		Anti-LPS titer	
		Total[b]	2-ME resistant[c]	Total[b]	2-ME resistant[c]
A	1	5.0	0.0	4.0	0.0
B	2	5.0	0.0	9.0	2.0
C	3	3.0	0.0	9.0	6.0
D	4	3.0	0.0	7.0	5.0

[a] An interval of 10 days was used between each injection of 50 μg of DNP-LPS. The mice were bled 4 days after the last injection of antigen.

[b] Titer of sera pooled from five mice per group.

[c] Titer of sera pooled from five mice per group after treatment with 2-ME.

TABLE 5. *Qualitative changes in the antibody responses of RML and nude mice injected repeatedly with LPS*

Group	Phenotype	No. of injections of LPS[a]	Antibody titer	
			Total[b]	2-ME resistant[c]
A	Normal	1	5.0	0.0
B	Normal	2	7.0	3.0
C	Normal	3	9.0	6.0
D	Normal	4	8.0	6.0
E	Nude	1	5.0	0.0
F	Nude	2	7.0	4.0
G	Nude	3	7.0	2.0
H	Nude	4	9.0	5.0

[a] An interval of 14 days was used between the first and second injections of 50 μg of LPS. The remaining injections were separated by intervals of 7 days. The mice were bled 4 days after the last injection of LPS.

[b] Anti-LPS titer of sera pooled from five mice per group.

[c] Anti-LPS titer of sera pooled from five mice per group after treatment with 2-ME.

were present in sera from both RML and nude mice injected two, three, or four times with LPS; the highest titers of 2-ME-resistant antibodies were found in sera of mice injected four times (Table 5). The presence of IgG antibodies in the serum of nude mice was shown also by separating IgM from IgG by density gradient ultracentrifugation and testing the separated fractions for anti-LPS hemagglutinating activity. Separation was verified with class-specific rabbit antisera to the mouse immunoglobulins. Antibodies to LPS were found in both the IgG and IgM fractions of antisera from normal (RML) and nude mice (Fig. 3). These data, combined with those above, showed that multiple injections of LPS stimulated the production of specific IgG antibodies in the absence of mature T cells.

DISCUSSION

DNP-LPS, administered as whole cells of *E. coli* O113, stimulated euthymic and athymic mice to produce only primary anti-DNP responses, whereas both primary and secondary anti-LPS responses were elicited by this conjugate. The failure of DNP-LPS to trigger secondary anti-DNP responses was not dependent on the primary or secondary dose of DNP-LPS used and was not influenced by the presence or absence of T cells. Furthermore, it could not be explained by a change in the class of antibody produced to DNP, combined with the relative lack of sensitivity of the passive hemagglutination assay to detect IgG antibodies. Repeated injections of mice with DNP-LPS could not elicit greater than primary anti-DNP responses. In other T cell-independent hapten-carrier systems, including trinitrophenylated LPS, only primary IgM anti-hapten responses were elicited, even after repeated injections of the homologous conjugate (7, 11). The present work showed that, in contrast to the anti-hapten response, primary and secondary responses were made against the determinants of the LPS carrier. Secondary anti-LPS responses were demonstrated by increased antibody titers, prolonged antibody synthesis, and synthesis of antibodies in the IgG class after multiple injections of DNP-LPS. Therefore, these data are in agreement with the results of Desagmard and Feldmann (8). A dissociation of the anti-hapten and anti-carrier responses was achieved after immunization with a hapten coupled to a T cell-independent carrier.

Different models, based on two immunological signals described by Bretscher and Cohn (5), have been proposed for the activation of B cells (15). Earlier reports showed a functional separation in the immunological signals required to stimulate primary and secondary antibody responses to polysaccharide antigens (31, 32). It was shown that one non-mitogenic antigen-specific signal was sufficient to stimulate a primary antibody response (29, 31, 32), whereas both an antigenic and a second nonspecific signal were necessary to evoke a secondary response (31, 32). In this context, DNP-LPS probably possessed both signals with respect to the LPS. Consequently, both primary and secondary anti-LPS responses were elicited by DNP-LPS. The finding that only primary anti-DNP responses were stimulated by DNP-LPS suggests that the nonspecific second signal associated with the cell wall LPS did not participate in the induction of anti-DNP responses by this conjugate. Therefore, the immunogenicity of the DNP portion of the DNP-LPS complex did not depend on the

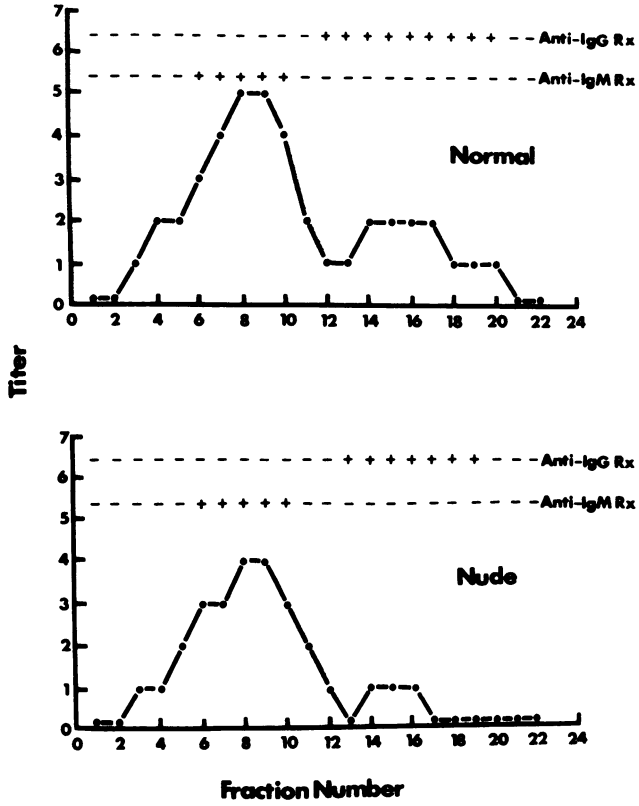

FIG. 3. *Presence of IgM and IgG antibodies specific for LPS in sera from normal and nude mice injected four times with LPS. The protocol followed for the immunizations and bleedings is described in footnote a of Table 5. The sera were centrifuged at 254,000 × g for 10 h at 4°C on a 10 to 50% discontinuous sucrose gradient. Fractions were collected from the bottom of the tubes and tested for hemagglutinating antibodies against LPS and for reactivity with rabbit anti-mouse IgM and rabbit anti-mouse IgG sera.*

second signal of the carrier. This observation is in agreement with several other examples of elicitation of primary T cell-independent responses in the absence of a second nonspecific signal (2, 16, 22, 23, 27, 31, 32).

Also of interest was the observation that nude mice injected repeatedly with LPS produced an IgG response to the LPS determinants. This finding confirms that of another article which reported that nude mice could be stimulated to synthesize specific IgG antibodies (21). Apparently some antigens, including LPS of *E. coli*, can circumvent the need for T cells in the activation of B cells which synthesize IgG.

In conclusion, the data reported herein, and in other published reports (27, 31, 32), led us to the following working hypothesis. LPS antigens, presented either as extracted LPS or as whole cells, possessed two activating signals. Both of these signals, administered in the appropriate manner, were necessary to activate B cells responsible for synthesizing IgG antibodies. Although the exact nature of the second signal is not known, it was thought to reside in or be dependent on the lipid A region of the LPS molecule. A second population of B cells was activated to produce IgM antibodies by a single antigen-specific signal, represented in our experiments with native protoplasmic polysaccharide (31), alkaline-detoxified LPS (32), LPS injected into C3H/HeJ mice (27), and DNP determinants of the DNP-LPS conjugate. Experiments with isolated B cell subpopulations and in vitro culture techniques are in progress and should provide more definitive information on the interaction of LPS antigens with different populations of B cells.

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